



PREVALENCE OF MULTIPLE ANTIBIOTIC RESISTANCE AMONG BACTERIA ISOLATED FROM WASTEWATER OF HOME-SITED AQUACULTURE IN OGUN STATE, SOUTH-WESTERN NIGERIA

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Manuscript received: 19/09/2018

Accepted: 11/01/2019

Published: March 2019

ABSTRACT

Antibiotics use in aquaculture has been reported to have significant impact on antibiotic selection pressure in wastewaters generated. There is a rising environmental and public health concern over its unhygienic disposal. This study investigated the prevalence of multiple antibiotic resistant bacteria in wastewaters of six aquaculture farms from Ijebu Ode, Ijebu Igbo and Ago Iwoye towns of Ogun State, South-western Nigeria. Bacteria isolation and the determination of microbial load were carried out by standard microbiological procedures. The susceptibility of isolates to ten antibiotics was tested for using disc diffusion method. Multiple Antibiotic Resistance (MAR) indexes was calculated. A total of 148 organisms were isolated belonging to nine genera of public health importance including *Pseudomonas*, *Serratia*, *Escherichia*, *Enterobacter*, *Citrobacter*, *Staphylococcus*, *Klebsiella*, *Proteus* and *Bacillus*. The microbial load ranged between 4.4×10^8 to 10.6×10^8 and 6.8×10^6 to 10.8×10^6 colony forming units (c.f.u./ml) for total heterotrophic count and total coliform counts respectively. Isolates showed varying degree of resistances to test antibiotics ranging between 0% for ciprofloxacin to 100% for ampicillin. The highest resistances were displayed to frequently administered antibiotics on the farms, (ampicillin 100%; oxytetracycline 94%; chloramphenicol 86.5%). MAR indices of all sites were above 0.2. Result of this study showed that aquaculture wastewaters can serve as MAR bacteria reservoirs whose unhygienic discharge may allow contamination of surrounding environment with consequent risk to human health.

Keywords: *aquaculture wastewater, Multiple antibiotic resistance, bacteria, antibiotic use.*

INTRODUCTION

The increasing surge in the emergence of antibiotic resistance among bacterial pathogens has become a daunting threat to effective clinical therapy with several factors being listed to account for its spread (WHO, 2014). Prominent among these factors is resistance development resulting from use of antibiotics in animal husbandry (Buschnann *et al.*, 2012). Aquaculture is widely practiced in the world; especially in developing countries where farmers lack knowledge of safe and proper use of antibiotics and respective Government agencies offer weak regulation on its use (Chuah *et al.*, 2016; FAO, 2016). It may be difficult to put a definite figure on global consumption of antibiotics in aquaculture. The world health organization gave a general estimate of half of the total antibiotic production being used up in veterinary medicine (WHO, 2014). In Chile an estimate of approximately 100-110 metric tons of quinolone was reported to have been consumed for human therapy and veterinary respectively with most of the animal consumption going into aquaculture (Cabello, 2013).

Unlike in human medicine where there is stricter use of antibiotics, the peculiarities of aquaculture permits the exposure of both sick and healthy aquatic lives to antibiotic dosing in the course of treating sick ones (Metaphylaxis) (Holmstrom *et al.*, 2003). Likewise, antibiotic is further abused by the use of small subtherapeutic dosing of antibiotics as a means to enhance growth. These activities uniquely contribute to the selection of resistance and resistance genes in aquaculture rearing waters (Burrige, 2012). The unlimited access to antimicrobials by farmer in developing countries such as Nigeria makes this even more worrisome, as farmers antibiotic use often extend beyond drugs stipulated for veterinary use.

Nigeria in recent years has witnessed a huge boom in the fish farming business. In addition to full time farmers who farm in secluded settlements away from the metropolis, a large number of people have taken up fish farming on part time basis which has led to springing up of fish farm in peoples' yards. Most home sited aquaculture farms rear under strict intensive method which makes greater demands on antibiotics for prophylaxis and treatment (Holmstrom *et al.*, 2003; Burrige *et al.*, 2010) Resistance development is often supported under high antibiotic pressures and the aquaculture wastewater provide a rather robust condition for this.

The discharge of untreated wastewater into a natural environment has been reputed to be a significant source of contamination in the environment. Spread of resistance determinants to aquatic and soil organisms, pollution of adjacent waters as well as propagation of resistant strains of pathogens have been reported as a

result of such practices (Adelowo *et al.*, 2009). The greatest environmental risks include the expansion of reservoir of resistant bacteria and antibiotic resistant genes when wastewater infiltrate into groundwater or washes into surface water. These transfers are facilitated by mobile genetic elements which may enhance their dissemination into phylogenetically diverse bacteria (Kummerer, 2009; Ganguly *et al.*, 2011). The siting of aquaculture has also been implicated in the contamination of its immediate environment. Boschmann *et al.* (2016) reported an increase in the number of antibiotic resistant bacteria present in sediment as a result of shrimp farming. In a similar investigation, very high prevalence of antibiotic resistant bacteria were reported from both primary aquaculture sites and adjacent environments of an aquaculture farm, mainly as a result of aquaculture practices (Shah *et al.*, 2014).

In a country as Nigeria where aquaculture farms are springing up in people backyard, notably lacking is information about the possible environmental impact especially their possibility of lending to reservoir of antibiotic resistant bacteria and ultimately being of health risk to the public. This study investigated the prevalence of multiple antibiotic resistant bacteria in the wastewaters of home sited fish-farms selected from Ijebu-Ode, Ijebu Igbo and Ago-Iwoye towns of Ogun State, South-western Nigeria to assess their status as environmental reservoirs of MAR bacteria.

MATERIALS AND METHOD

Six aquaculture farms were selected for this study in Ijebu-Ode, Ijebu- Igbo and Ago-Iwoye towns in Ogun State of South-western Nigeria (Table 1). The towns are urban but with an appreciable level of agricultural practices within the town and in agricultural settlements. Inhabitants of these towns practice aquaculture on both large and small scales. The six study farms kept multiple ponds and were located within the metropolis. In most of the farms that rear in concrete ponds, wastewater generated were simply linked to poorly constructed public drainage system, mostly open ended which allows it to spill over onto the ground, otherwise the disposal was done towards surface water within the vicinity. No treatment was given to the wastewater prior to discharge. Interactions with the farmers revealed that antibiotics were in use in all of the six farms; the most popular of these were neomycin, oxytetracycline and penicillin (Table 1). Their use, as volunteered by the farmers interviewed on sites, was for prophylactic and infectious disease management. It was obvious that none of these farmers had adequate knowledge on safe and correct application of these antibiotics. In all of the farms,

no standard antibiotic dosage was followed although drug packs carry the prescribed dosing. Antibiotics were simply applied by pouring into ponds by hand or being mixed with fish pellets.

Samples of wastewater were collected between December and April, 2017. Aquaculture wastewaters were collected at the time of pond-water change for a period of 4 months in intervals of 3 weeks. Composite sampling was done as each of the farms had multiple ponds, 50ml volumes of wastewater were collected from discharge pipes into sterile containers. The pooled samples were transported to the laboratory in ice buckets for the isolation of bacteria within eight hours of collection. 1ml of the composite sample was drawn and serially diluted to 10th dilution. A 0.2ml aliquot of appropriate dilution was then spread on the surface of already prepared nutrient agar, MacConkey, EosinMethylene blue (EMB), Xylose Lysine Deoxycholate (XLD) agar (OXOID, Basingstoke, UK) plates for the isolation of bacteria.

These plates were incubated aerobically at 37°C for 24hours. Further sub-culturing followed until morphologically distinct colonies were obtained. The identity of the colonies was determined by standard methods (Barrow and Feltham, 2003).

The susceptibility of bacterial isolates from the aquaculture wastewaters to ten selected antibiotics was tested for using the disc diffusion method (Bauer *et al.*, 1966). Sensitivity discs impregnated with the following antibiotics; neomycin 10µg, erythromycin 15µg, oxytetracycline 30µg, ampicillin 10µg, streptomycin 10µg, chloramphenicol 30µg, ceftazidime 30µg, ciprofloxacin 5µg and Sulfamethoxazole/trimetoprim were used. The choice of antibiotics used in this study was based on their relevance to aquaculture production and human clinical medicine in Nigeria. Inoculums were prepared by suspending well isolated, morphologically distinct bacterium into 4ml sterile distilled water. These were then adequately adjusted to a 0.5 McFarland turbidity standard. The resulting suspensions were used to inoculate already prepared plates of Mueller Hinton agar using sterile swab-sticks and respective antibiotic sensitivity disks were layered within 15minutes of inoculation. Plates were incubated aerobically at 37°C for 24 h. Thereafter, zones of growth inhibition around each disc was measured and appropriately interpreted following the Clinical Laboratory Standard Institute guidelines (CLSI, 2012). Data obtained were used to construct the phenotypic pattern of drug resistance for each organism. The antibiotic resistance indices of the study sites were calculated according to the method of Krumpfenhagen, 1983.

Results were analyzed by descriptive statistics using the statistical software SPSS version12.0.

RESULTS and DISCUSSION

A total of one hundred and forty eight bacteria were isolated from aquaculture wastewaters in this study. Sixty eight isolates which correspond to 43.0% were recovered from the aquaculture wastewaters of Ijebu Igbo ponds designated AqFa and AqFb respectively. Fifty one, (42.3%) from two Ago Iwoye ponds designated AqFc and AqFd; while n=39 (24.7%) isolates were recovered from Ijebu Ode, designated AqFe and AqFf (Table 1).

Table 1: Description of activities of study sites

Site codes	Pond Type	Number Of ponds	Antibiotics Consumed	Cultured Species	Mode of Effluent Disposal
AqFa	Earthen Ponds	5	Streptomycin	Tilapia Cat Fishes	Disposed into surface Water
AqFb	Concrete Ponds	7	Neomycin Oxytetracycline Erythromycin	Cat Fishes	Disposed on the ground
AqFc	Concrete Ponds	6	Oxytetracycline Erythromycin	Cat Fishes	Disposed on the ground
AqFd	Concrete Ponds	5	Procaine and Benzyl Penicillin	Cat Fishes	Disposed into surface Water
AqFe	Concrete Ponds	8	Cloramphenicol Neomycin Oxytetracycline Furaltadone	Cat Fishes	Disposed into the drainage
AqFf	Concrete Ponds	2	oxytetracycline	Cat Fishes	Disposed into unlined drainage

AqFa, AqFb: aquaculture farms sited at Ijebu Igbo

AqFc, AqFd: aquaculture farms sited at Ago Iwoye; AqFe, AqFf: aquaculture farms sited at Ijebu Ode.

Isolated organisms belonged to nine genera of public health concern namely, *Pseudomonas*, *Serratia*, *Escherichia*, *Enterobacter*, *Citrobacter*, *Staphylococcus*, *Klebsiella*, *Proteus* and *Aeromonas* spp. (Table 2). Generally, the most frequently isolated genera were *Enterobacter*, *Aeromonas* and *Escherichia coli*, (detected in all sampled wastewaters) closely followed by *Citrobacter* spp. The isolation of these organisms from studied wastewaters may have health implications for inhabitants of the areas. *Enterobacter* spp. and *Escherichia coli* which were consistently present in the wastewaters are primarily considered as indicators of fecal pollution capable of affecting human health via contamination of fish and surrounding drinking water sources (Surendraraj *et al.*, 2009).

Table 2: Distribution of isolated organisms in the three sampled towns

Sampling towns	Organisms	Number of Isolates
Ijebu Igbo	<i>Pseudomonas aeruginosa</i>	10
	<i>Escherichia coli</i>	18
	<i>Klebsiella sp.</i>	4
	<i>Enterobacter sp.</i>	8
	<i>Proteus vulgaris</i>	3
	<i>Salmonella sp.</i>	3
	<i>Citrobacter sp.</i>	12
	<i>Staphylococcus aureus</i>	5
	<i>Serratiamascecens</i>	3
	<i>Bacillus sp</i>	1
	<i>Aeromonas sp.</i>	1
Ago Iwoye	<i>Bacillus subtilis</i>	1
	<i>Enterobacter sp.</i>	7
	<i>Staphylococcus aureus</i>	2
	<i>Citrobacter sp</i>	11
	<i>Escherichia coli</i>	13
	<i>Proteus vulgaris</i>	4
	<i>Pseudomonas aeruginosa</i>	8
	<i>Aeromonas sp</i>	3
	<i>Serratia sp</i>	2
Ijebu Ode	<i>Citrobactersp.</i>	6
	<i>Salmonella sp</i>	6
	<i>Escherichia coli</i>	11
	<i>Pseudomonas aeruginosa</i>	4
	<i>Klebsiella sp.</i>	3
	<i>Proteus sp.</i>	3
	<i>Bacillus cereus</i>	1
	<i>Enterobacter sp.</i>	5

Amp, ampicillin; Str, streptomycin; OT, oxytetracycline; E, Erythromycin; C, chloramphenicol; Cip, ciprofloxacin; Caz, ceftazidime; ; Sxtsulphamethoxazole/trimetoprim., N Neomycin, Amc Amoxicillin/Trimetoprim

Aeromonas species, commonly associated with fish farm environment are gradually assuming considerable public health interest. An array of virulence factors have been reported in these bacteria making them important emerging human pathogens (Janda and Abbott, 2010; Hu *et al.*, 2012)

The microbial loads of aquaculture wastewaters being disposed were found to be generally high. It ranged from 4.4×10^8 to 10.6×10^8 cfu/ml for the total heterotrophic count (THC) and 6.8×10^6 to 10.8×10^6 cfu/ml for total coliform count (TCC). The highest microbial loads were from the earthen farm (AqFa) in Ijebu Igbo. The high microbial load is a reflection of the unhygienic status of aquaculture wastewaters

being disposed into the environment. Aquaculture wastewater is very rich in nutrient as it contain high amount of uneaten food and fish faeces which may lend to bacteria proliferation in the rearing water. The proximity of these aquaculture farms to groundwater sparks a concern for public health as ponds under study are sited within the metropolis with some located in proximity to surface water (streams). One of the farms in this study disposes into nearby stream, although this may not impact negatively via drinking, such water body may serve as reservoirs of pathogenic organisms and foster resistance exchange and spread. Other studies implicating aquaculture in contamination of its immediate environment include Boschmann *et al.*, 2012, Shah *et al.*, 2017; Igbinosa *et al.*, 2017.

Antibiotic resistances of isolated organisms to test antibiotics ranged between 0% to ciprofloxacin and neomycin and 100% to ampicillin. Within this margin, isolates displayed very high level of resistances to other antibiotics such as oxytetracycline (n=140) 94.5%, chloramphenicol 86.5%, erythromycin (n=131) 88.5%, Sulfamethoxazole/Trimetoprim (n=81) 56.7%. All were universally sensitive to ciprofloxacin; likewise to neomycin but for three isolates from AqFb in Ijebu Igbo that displayed intermediate resistance to it (Fig1). The high outcome in antibiotic resistance observed in this study may not be totally removed from the wide usage of antibiotics at the sampled farms; antibiotics are applied at one time or another during fish farming operations.

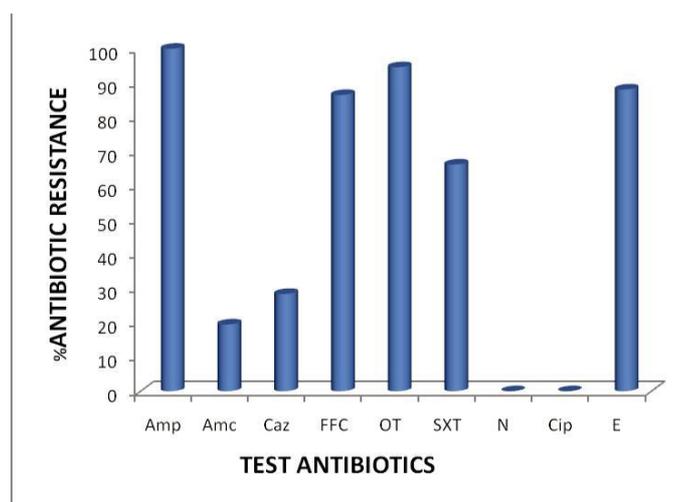


Fig 1. Antibiotics resistance patterns of isolated organisms to test antibiotics

Furthermore, isolates were observed to be highly resistant to most of the commonly used drugs on the farm; ampicillin 100%, oxytetracycline 94.6% and chloramphenicol 86.5%. Wrong usage of antibiotics have been clearly indicated in resistance development

of aquaculture site and its environment (Burrige *et al.*, 2011; Cabello, 2013). Results in this study are not completely dissimilar to previous reports of high antibiotic resistance patterns in aquaculture wastewater. In a study on Enterococci species from an aquaculture wastewater, Igbiosa *et al.* (2016) reported higher resistance patterns to test antibiotics. Similarly, *Escherichia coli* from aquaculture and its immediate environment were found to display high resistance to chloramphenicol (Ng *et al.*, 2014). This study found 0% resistance to neomycin in spite of its popular use (a particular drug named aquaceryl plus containing neomycin and oxytetracycline was popularly used in two of the farms). However, very high resistance was displayed to the associated antibiotic; oxytetracycline. Organisms displayed varying degree of multiple antibiotic resistances. Sixty four isolates from Ijebu Igbo were MAR to at least three unrelated antibiotic classes. This was closely followed by 37 (72.5%) and 21 (56.7%) isolates from Ago Iwoye and Ijebu Ode respectively. Two strains of *Pseudomonas aeruginosa*, two *Escherichia coli* from Ijebu Igbo and one *Aeromonas* sp from Ijebu Ode were resistant to eight of the ten test antibiotics. Sixteen phenotypic patterns of drug resistance were observed among the fifty one isolates from Ago Iwoye. Isolates from Ijebu Igbo displayed fourteen notable phenotypic patterns while ten patterns were observed among isolates in Ijebu Ode (Table 3).

Table 3: List of antibiotics to which isolated organisms developed resistance

S/No	Antibiotic resisted	Number of Resistant Organisms
Ijebu Igbo	Amp.	4
	Amp. E.	4
	Amp. Ot	7
	Amp. Ot. E.	17
	Amp. Caz. C. Sxt. Ot	5
	Amp. C. E. Sxt. Ot	3
	Amp. S. E. Sxt. Ot	3
	Amp. C. E. Sxt. Ot	1
	Amp. Caz. E. Sxt. Ot	5
	Amp. Caz. C. E. Sxt. Ot	4
	Amp. Amc. Caz. C. S. E. Sxt. Ot	3
	Amp. Amc. Caz. C. E. Sxt. Ot.	3
	Amp. Caz. Amp. C. S. E. Sxt.Ot.	1
	Amp, Amc, Caz, C, S, E, Ot	2
Ago Iwoye	Amp.	5
	Amp. Ot.	1
	S.E.	1
	Amp. E.	3
	Amp. E. O. Caz. Amc. C	1

	Amp. Caz. Sxt. C	1
	Amp. Sxt. C. E. Ot.	1
	Amp. Caz. Sxt. C. E. Ot.	1
	Amp. Sxt. C. E. Ot.	4
	Amp. S. E. Sxt. C. Ot	9
	Amp, Sxt, C, E	1
	Amp. Sxt. E. S. C. Ot	7
	Amp, Caz, Amc, C, E, Ot	5
	Amp. Caz, Amc, Sxt, C, E. Ot.	4
	Amp. Caz. Sxt. C. S. E. Ot.	2
	Amp. Caz. Amc. Sxt. C. E. S. Ot.	4
Ijebu Ode	Amp	8
	Amp.Ot.	2
	Amp. S.	3
	Amp.E.Ot.	5
	Amp.	3
	Amp. Sxt. E. C. Ot	4
	Amp, Caz, Amc, E. Ot	3
	Amp, Caz, Amc, E. Ot	4
	Amp. Sxt. E. S. Ot	3
	Amp. Caz. Sxt.S.E.Ot	2

Amp, ampicillin; Str, streptomycin; OT, oxytetracycline; E, Erythromycin; C chloramphenicol; Cip, ciprofloxacin; Caz, ceftazidine; ; Sxt sulphametoxazole/trimetoprim.

Multiple antibiotic resistance indices of the sampled sites revealed that all the aquaculture environments had MAR index above acceptable limits of (0.2). It ranged between 0.24 and 0.63. Studies have reported the significance of aquaculture environment as important resistomes which actively drive the transfer of antibiotic resistant genes (Cabello *et al.*, 2006; Akinbowale *et al.*, 2007; Cabello *et al.*, 2013). In a related study, Adelowo *et al.* (2014), described food animals and their respective rearing environments as reservoirs of resistant bacteria and antibiotic resistant genes which are capable of being taken up via direct or indirect means with eventual consequence on human health. The Multiple antibiotic resistance indices of aquaculture wastewater sites in this study were all found to exceed the acceptable limit (0.2), the highest was from the Ijebu Igbo sites and the least being 0.24 from Ijebu Ode. The MAR index, which ranged from 0 to 1.0 is used as a tool for analyzing the risk assessment of an environment exposed to use of antibiotics. The high MAR indices in this study may be a clear reflection of the antibiotics driven selective pressure in the studied sites.

Table 4. Multiple Antibiotic Resistance Indices of the different Aquaculture sites

Aquaculture sites	Multiple Antibiotic resistance (MAR) Index
AqFa	0.63
AqFb	0.44
AqFc	0.24
AqFd	0.38
AqFe	0.32
AqFf	0.34

AqFa, AqFb: aquaculture farms sited at Ijebu Igbo
 AqFc, AqFd: aquaculture farms sited at Ago Iwoye
 AqFe, AqFf: aquaculture farms sited at Ijebu Ode

Furthermore, environments with antibiotic residues can exert antibiotic pressure long after seizure of fish farming in a particular area (Chuah *et al.*, 2016). A fact that calls for the routine examination of surrounding environs of aquaculture sites, to monitor impact.

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CONCLUSION

The present study revealed that aquaculture wastewater can serve as an environmental reservoir of multiple antibiotic resistant bacteria. These reservoirs are potential route of contamination of the aquaculture environments itself and particularly human population with resistant bacteria and the consequences on clinical therapy is grave. Attention is drawn to unregulated use of antibiotics for animal production, springing up of aquaculture farms within the metropolis and discharge of untreated wastewater into the environment in the global efforts to combat antibiotic resistance. Consequent to the findings in this study, work is ongoing in examining surface and groundwater around these aquaculture farms to determine their status as reservoirs of multiple antibiotic resistance and molecular characterization of such isolates to establish their relatedness to wastewater strains.

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